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(54) Title: INHIBITING UNWANTED IMMUNE RESPONSES

(57) Abstract

A hybrid protein including protein fragments joined together by covalent bonds, the protein fragments including: a fragment including a portion of IL-2, the portion including at least a portion of the binding domain of IL-2, the portion of the binding domain being effective to cause the hybrid protein to bind selectively to cells bearing the IL-2 receptor; and, an enzymatically inactive fragment of diphtheria toxin which does not include a functional diphtheria toxin generalized eukaryotic binding site. The hybrid protein capable of stimulating the proliferation of PBMC *in vivo* and of suppressing an immune response in a mammal *in vivo*.

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INHIBITING UNWANTED IMMUNE RESPONSES

Background of the Invention

This invention relates to the inhibition of unwanted immune responses, e.g., as occur in autoimmune diseases and in the rejection of allografts.

Autoimmune diseases are characterized by an immune response which is directed against constituents of a patient's own tissues and which damages those tissues. These disorders arise, at least in part, from the defective regulation of the immune system by T-cells, see generally, Robinson (1990) in Medicine, Scientific American, New York, New York, pp. 6:VI:1-10. T-cells play a central part in the propagation of an immune response. Helper-inducer-T-cells specific for an antigen stimulate the appropriate B-cells to produce antibody and specific suppressor T-cells inhibit B-cells from producing antibody. The activity of T-helper-cells is dependent on interactions with monocytes, macrophages, B-cells, and other lymphocytes, Haynes et al. (1986), in Harrison's Principle of Internal Medicine, 11th Ed. Mcgraw-Hill, New York. Monocytes and macrophages activate T-cells by secreting interleukin 1, which induces the expression of receptors for the T-cell derived lymphokine interleukin 2 (IL-2). IL-2 results in T-cell proliferation and differentiation, the activation of cytotoxic T-cells, and the stimulation of B-cell responses, Kehrl et al. (1984) Immunol. Rev. 78:75, Lehti et al. (1986) J. Clin. Invest. 77:1173.

The critical role of T-lymphocytes in the function of the immune system in autoimmune disease is suggested in experiments with the drug cyclosporine. Cyclosporine inhibits the proliferation of helper-inducer and cytotoxic T-cells, but does not inhibit antigen-induced activation of suppressor T-cells, and thus suppresses

immune responses, Cohen, et al. (1984) Ann. Intern. Med. 101:667. The administration of cyclosporine to rats with experimentally induced myasthenia gravis (induced by the injection of purified acetylcholine receptor) has been shown, in vitro, to decrease the severity of the induced autoimmune disease. Production of anti-acetylcholine receptor antibody, but not of unrelated antibodies, was inhibited when lymphocytes from such rats were incubated with cyclosporine and acetylcholine receptor, MacIntosh, et al. (1986) Science 232:401. Thus a substance which is known to interfere with T-cell immunoregulation interrupts the disease process in an experimentally induced autoimmune disease.

Two of the most common serious autoimmune diseases are diabetes and rheumatoid arthritis, see Table 1, which lists autoimmune diseases and their characteristic antigens. Juvenile insulin-dependent diabetes is an autoimmune disease in which the immune system directs a response against the cells of the islets of Langerhans. These cells, which are located within the pancreas, are responsible for producing insulin. As a result of the autoimmune response the islet cells are impaired or destroyed, resulting in insulin deficiency.

The chief autoantigen of rheumatoid arthritis is believed to be Type 2 collagen. Type 2 collagen is a structural protein found in the joints and in the vitreous tissue of the eye, Bornstein (1980) Ann. Rev. Biochem. 49:957. The autoimmune response characteristic of rheumatoid arthritis leads to destructive processes in the joints.

Rheumatoid arthritis is perhaps the most well studied autoimmune disease, in part because of the existence of two inducible animal models, collagen arthritis (CA) and adjuvant arthritis (AA). CA can be

induced in rats, Trentham et al. (1977) J. Exp. Med. 146:857, mice, Courtenay et al. (1980) Nature 283:666, or monkeys, Cathcart et al. (1986) Lab. Invest. 54:26, by an intradermal injection of native Type 2 collagen emulsified in oil, Stuart et al. (1979) Arthritis Rheum. 22:347, Stuart et al. (1984) Ann. Rev. Immunol. 2:199, Trentham (1984) Proc. Soc. Exp. Biol. Med. 176:75. AA, which can only be established in rats, is induced by an injection of oil containing a preparation that possesses adjuvant activity, i.e., a preparation that contains heat killed and desiccated Mycobacterium tuberculosis.

A number of lines in evidence from work with these animal models demonstrate the importance of the cellular immune response in autoimmune disease. T-lymphocytes play a prominent role in the progress of both CA and AA. In animals afflicted with CA the majority of the mononuclear cells infiltrating the synovium during the inceptual phase of arthritis belong to the T helper subset, based on their display of the W3/25 epitope, while T-non-helper-lymphocytes dominate in the later phases of the disease, Holmdahl et al. (1985) Scand. J. Immuno. 21:197. Furthermore, thymus deficient rats are refractory to the induction of CA, Klareskoy (1983) Clin. Exp. Immunol. 51:117, and CA can be prevented by administration of either anti-thymocyte antibody or monoclonal antibodies directed against an antigen found on murine T-helper-cells, Brahn et al. (1984) Cell Immunol. 86:421, Ranges et al. (1985) J. Exp. Med. 162:1105. Other experiments provide evidence for the combined participation of T and B cells in the CA disease process, e.g., pretreatment of rats with anti-μ serum, a procedure which interferes with B cell maturation, inhibits the onset of CA.

T-cells also play a dominant role in the progress of AA, as illustrated by the requirement of an intact

thymus for the induction of the disease, Kohaski et al. (1981) Infect. Immun. 31:758, and by the suppressible nature of the disease by anti-lymphocyte antibodies, Currey et al. (1968) J. Exp. Med. 127:185.

A fundamental role for T-cells in the autoimmune response is also supported by observations that a T-cell response to Type 2 collagen can be demonstrated in vitro in many patients with rheumatoid arthritis, Trentham et al. (1978) New Eng. J. of Med. 299:327.

Allograft rejection is an immune response involving activated T-lymphocytes. Current immunosuppressive protocols designed to inhibit rejection of allograft-tissue involve the administration of drugs such as azathioprine, cyclosporine, and corticosteroids, all of which cause toxic side-effects to non-lymphoid tissues. The recent development of pan-T-lymphocyte monoclonal antibodies represents an important refinement in therapy, since only T-lymphocytes are targeted by the administration of such antibodies. However, this therapy has the disadvantage of destroying, along with the T-lymphocytes involved in allograft rejection, those required for normal immune surveillance.

Selective immunosuppression has been achieved using the chimeric toxin DAB_{486}-IL-2, which includes the cytotoxic portion of diphtheria toxin fused to interleukin 2, (IL-2), Williams et al. (1987) Protein Engineering 1:493-498, hereby incorporated by reference, to specifically target and kill cells bearing the high-affinity IL-2 receptor, Waters et al. (1990) Eur. J. Immunol. 20:785-791. During the proliferative burst following antigen recognition, T-cells express cell-surface high-affinity IL-2 receptors. Targeted destruction of the activated cells bearing high affinity IL-2 receptors with the DAB_{486}-IL-2 chimeric toxin

inactivates an ongoing immune response while sparing the majority of the cells in the host's immune repertoire.

Summary of the Invention

In general, the molecules and methods of the invention provide for the inhibition of an unwanted immune response. The methods involve administering to a mammal an inhibiting amount of a hybrid protein including protein fragments joined together by covalent bonds, the protein fragments including: a fragment including a portion of IL-2, the portion including at least a portion of the binding domain of IL-2, the portion of the binding domain being effective to cause the hybrid protein to bind selectively to cells bearing the IL-2 receptor (examples of IL-2 deletions that are capable of binding to the IL-2 receptor are found in Genbauffe et al., U.S.S.N. 388,557, filed August 2, 1989, and hereby incorporated by reference); and, an enzymatically inactive fragment of diphtheria toxin which does not include a functional diphtheria toxin generalized eukaryotic binding site. The hybrid protein is capable of stimulating the proliferation of peripheral blood mononuclear cells (PBMC) in vitro and of suppressing an immune response in vivo.

In preferred embodiments, the fragment of the diphtheria toxin molecule has been enzymatically inactivated by a mutation, preferably a mutation at position 53 of the diphtheria toxin.

Any portion of diphtheria toxin which does not include a functional generalized eukaryotic binding site, and which when fused to IL-2 is capable of: 1) in vitro stimulation of PBMC; and 2) suppression of an immune response in vivo, can be used in the methods and molecules of the invention. Determination of whether a hybrid protein satisfies these two qualifications is, in light of the guidance provided below, a routine matter

for one skilled in the art. In preferred embodiments the inactive fragment includes residues 1-485 of diphtheria toxin, although not all of the amino acids upstream to residue 485 need be included. Preferably one or more of: domain l_2; the hydrophobic transmembrane region; Fragment A; Fragment B; or the protease sensitive domain, is deleted. In other preferred embodiments at least 10%, more preferably at least 20%, and most preferably at least 30% of the amino acid residues between positions 1 and 485 of diphtheria toxin are deleted.

The invention includes a DNA sequence encoding the hybrid protein of the invention, an expression vector containing that DNA sequence, a cell transformed with that vector, and a method of producing the hybrid protein including culturing the cell and isolating the hybrid protein from the cultured cell or supernatant.

The methods of the invention also include: a method of inhibiting unwanted immune response in a mammal, including administering to the mammal an inhibiting amount of the hybrid protein; a method of inhibiting the T-lymphocyte- induced rejection of an allograft in a mammal, including administering to the mammal, following the allograft, and during the proliferative burst, a hybrid protein or the invention; and, a method of treating a patient having a disease, e.g., an autoimmune disease, characterized by a proliferative burst of lymphocytes, including administering to the patient, during the proliferative burst, a hybrid protein of the invention.

Enzymatically inactive diphtheria toxin, as used herein, means a diphtheria toxin molecule which has been modified, e.g., by mutation or by chemical modification, such that when present in the hybrid protein of the invention it no longer possesses sufficient ADP-ribosyl

transferase activity to kill cells by virtue of ADP-ribosyl transferase activity.

In vitro stimulation of PMBC, as used herein, means an increase in any of: DNA synthesis; protein synthesis; or, of the secretion of a growth factor, e.g., a cytokine.

In vivo suppression of an immune response, as used herein, means any of: a decrease in delayed type hypersensitivity (DTH); a decrease in an immune response directed against transplanted tissue or a transplanted organ, manifest, e.g., by a delay in rejection of the transplanted tissue or organ; a decrease in the symptoms of an autoimmune disease; or an in vivo decrease in any other immunologic parameter known to one skilled in the art as indicative of the extent of an immune response.

Specific binding, as used herein, refers to the ability of a substance to bind virtually exclusively to a particular growth factor receptor, e.g., to the IL-2 has a specific affinity for the IL-2 receptor, it binds IL-2 receptor and not to other cell surface receptor proteins, e.g., insulin receptors.

Diphtheria toxin, or native diphtheria toxin, as used herein, means the 535 amino acid residue mature form of diphtheria toxin protein secreted by Corynebacterium diphtheriae. The sequence of an allele of the gene which encodes native diphtheria toxin can be found in Greenfield et al. (1983) Proc. Natl. Acad. Sci. USA 80:6853-6857, hereby incorporated by reference. Enzymatically active Fragment A, as used herein, means amino acid residues Gly 1 through Arg 193 of native DT, or an enzymatically active derivative or analog of the natural sequence. Cleavage domain l_1, as used herein, means the protease sensitive domain within the region spanning Cys 186 and Cys 201 of native DT, Fragment B, as used herein, means the region from Ser 194 through Ser

535 of native DT. The hydrophobic transmembrane region, or hydrophobic domain, of Fragment B, as used herein, means the amino acid sequence bearing a structural similarity to the bilayer-spanning helices of integral membrane proteins and located approximately at or derived from amino acid residue 346 through amino acid residue 371 of native diphtheria toxin. Domain 1_2, as used herein, means the region spanning Cys 461 and Cys 471 of native DT. The generalized eukaryotic binding site of Fragment B, as used herein, means a region within the C-terminal 50 amino acid residues of native DT responsible for binding DT to its native receptor on the surface of eukaryotic cells. The generalized eukaryotic binding site of Fragment B is not included in the chimeric toxins of the invention.

The method of the invention inhibits an unwanted immune response as seen e.g., in autoimmune disease or in organ or tissue transplants, in a manner which does not cause general immune suppression, with its resulting risk of life-threatening infections. In addition, in the case of the treatment allograft rejection, the method spares donor-specific T suppressor cells, which can thus proliferate and aid in prolonging allograft survival. Furthermore, molecules of the invention do not need to be tailored to individual patients; a single specific ligand-blocking agent can be used as a universal inhibiting agent. In addition, therapy need not be continuous following allograft or following an acute stage of autoimmune diseases, but can be discontinued after a course of treatment.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first be briefly described.

Drawings

Fig. 1 is a representation of the coding sequence, and the corresponding amino acid sequence, for the IL-2 gene portion of plasmid pDW15, following SphI digestion of pDW15.

Fig. 2 is a diagram illustrating the construction of plasmid pSI130, which contains DNA encoding the DA(197)B_{486}-IL-2 fusion gene.

Fig. 3 is a diagram of the structure of DT.

Fig. 4 is a graph of the effect of DA(197)B_{486}-IL-2 on AA.

Fig. 5 is a graph of the response of DA(197)B_{486}-IL-2 treated mice to delayed hapten challenge.

Fig. 6 is a graph of the response of DA(197)B_{486}-IL-2 treated mice to rechallenge with a hapten.

Fig. 7 is a graph of the response of DA(197)B_{486}-IL-2 treated mice to challenge with a second hapten.

Fig. 8 is a graph of the effect of DA(197)B_{486}-IL-2 on ^{3}H-thymidine uptake in PBMC.

Construction of DA(197)B_{486}-IL-2

The procedure described below results in a chimeric gene encoding an enzymatically inactive diphtheria toxin molecule fused to IL-2. The IL-2 gene used for these fusions (Fig. 1) was obtained from plasmid pDW15 (Williams et al. (1988) Nucleic Acids Res. 16:10453, hereby incorporated by reference). pDW15 contains a synthetic form of the IL-2 gene which, when cloned into E.coli JM101, expresses IL-2 protein at a rate about 16 times that of the native cDNA sequence cloned into the same strain of E.coli. DNA encoding CRM197 was obtained from plasmids pß197 and pABJ6508, as described below.

The genetic fusion was made at the SphI site of pDW15 so that the IL-2 domain of the fused gene would encode 133 amino acids of IL-2, plus one additional amino acid on its amino terminus encoded by the Sph site (Fig. 1).

The construction of plasmid pSI130, which contains the gene encoding the CRM197 mutant of diphtheria toxin fused to IL-2, is illustrated in Fig. 2. Plasmid pABM6508, containing a gene coding for the N-terminal 485 amino acids of diphtheria toxin joined to α-melanocyte-stimulating hormone (Bishai et al. (1987) J. Bacteriol. 169:5140, hereby incorporated by reference), was digested completely with HindIII and partially with SphI and a 6kB SphI - HindIII vector fragment recovered. Plasmid pDW15 was digested with HindIII and with SphI and a 0.5kB SphI - HindIII IL-2-gene containing fragment recovered. The 6kB SpHI - HindIII fragment from pAMB6508 was ligated to the 0.5 kB SphI - HindIII fragment from pDW15 to produce plasmid pABI6508. pABI6508 was modified to include a lacIq promoter, and a gene for canamycin resistance in place of a gene for ampicillin resistance, using standard techniques. The modified pABI6508 was designated pSI100. pSI100 was digested with AccI and XmnI and a 5.1 kB vector fragment isolated. Plasmid pß197 (Bishai et al.) was digested with AccI and XmnI and a 0.65kB fragment isolated. pß197 carries the CRM197 mutant allele of DT, CRM197 is a full-length (535 amino acids) missense mutant (Gly_{52}-> Glu_{52}) whose protein product is devoid of ADP-ribosyl transferase activity and is thus nontoxic (Uchida et al. (1973) J. Bio. chem. 248:3838-3844. The missense mutation occurs within the 0.65kB AccI - XmnI fragment of pß197. The 5.1kB AccI - XmnI fragment from pSI100 was ligated to the 0.65kB AccI - XmnI fragment from pß197 to yield plasmid pSI130.

Expression of pSI130 in E.coli was induced as described by Bishai et al.; the CRM197/IL-2 gene product was purified using affinity chromatography (Williams et al.) followed by HPLC size exclusion chromatography. (Methods in Enzymology (1983) 91:137, hereby incorporated by reference).

Standard methods for the manipulation of DNA, well known to those skilled in the art, were employed. Plasmids were introduced into E.coli by $CaCl_2$ transformation, isolated by the alkaline lysis procedure, and purified by CsCl density gradient centrifugation (Maniatis et al., Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982, hereby incorporated by reference).

Alternatively, Plasmid DNA was purified by the alkaline lysis/ cesium chloride gradient method of Ausebel et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., hereby incorporated by reference. DNA was digested with restriction endonucleases as recommended by the manufacturer (New England Biolabs, Beverly, MA and Bethesda Research Laboratories, Gaithersburg, MD). Restriction fragments for plasmid construction were extracted from agarose-TBE gels, ligated together (with or without oligonucleotide linkers) and used to transform E. coli using standard methods. Ausebel et al (1989) supra and Maniatis et al. (1982), Molecular Cloning Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., hereby incorporated by reference. Plasmid DNA sequencing was performed according to the dideoxy chain termination method of Sanger et al. (1987) Proc. Natl. Acad. Sci USA 74:5463-5467, hereby incorporated by reference, as modified by Kraft et al. (1988) Bio Techniques 6:544-547, hereby incorporated by reference, using Sequenase (United States Biochemicals, Cleveland, OH).

Construction of chimeric toxin DAB_{486}-IL-2

DAB_{486}-IL-2 is a chimeric toxin consisting of Met followed by amino acid residues 1 through 485 of mature DT fused to amino acid residues 2 through 133 of IL-2. The DT portion of the chimeric toxin DAB_{486}- IL-2 includes all of DT fragment A and the portion of DT fragment B extending to residue 485 of mature native DT. Thus DAB_{486}-IL-2 extends past the disulfide bridge linking Cys 461 with Cys 471. See Fig. 3 for the structure of DT.

The sequence of DT is given in Greenfield et al. (1983) supra. The sequence encoding IL-2 was synthesized on an Applied Biosystems DNA-Synthesizer, as described in Williams et al. (1988) Nucleic Acids Res. 16:10453-10467, hereby incorporated by reference. The sequence of IL-2 is found in Williams et al. (1988) Nucleic Acids Res. 16:10453-10467, and was linked to the DT sequences as described in Williams et al. (1987) supra. The DT signal sequence was removed and fused to the sequence encoding mature DT which was fused to ATG using an oligonucleotide linker as described in Bishai et al. (1987) J. Bact. 169:5140-5151.

DA(197)B_{486}-IL-2 and DAB_{486}-IL-2 are Anti-Arthritogenic

Selected methods and molecules of the invention provide therapy for autoimmune diseases, e.g., arthritis. AA is a rat model of the human T-cell mediated autoimmune disease, rheumatoid arthritis. First recognized approximately three decades ago, Pearson (1956) Proc. Soc. Exp. Bio. Med. 91:95, AA appears as a subacute inflammation which progresses to a chronic polyarthritis. Although AA is triggered by an immune response to mycobacterial antigens, the disease is transferable to naive rats by injection of lymphoid cells from affected rats, Pearson (1964) J. Exp. Med. 120:547. AA is inducible in genetically susceptible rats

(Lewis rats) by intradermal injection of Complete Freund's Adjuvant (CFA). In certain strains of rats the incidence rate for development of the disease is 100%. The induced arthritis, which appears in approximately 11-13 days after injection with CFA, is prominent in the small joints of the extremities and is characterized by inflammation of the joints and erosion of the bone. Affected animals become progressively more disabled until approximately day 20 - day 25 post-immunization with CFA. Clinical symptoms gradually decrease to plateau at a level which is approximately 50% of the peak. They do not spontaneously remit. AA resembles rheumatoid arthritis in the pathologic and physiologic features of pannus formation, synovitis, mononuclear cell infiltration of the synovium, destruction of the cartilage, and erosion of bone.

The effect of the hybrid protein DA(197)B_{486}-IL-2 on AA is shown in the following experiment.

Thirty female Lewis rats (weighing approximately 125g) were divided into three groups. On day -1 through day 9 the animals in group I (solid line in Fig. 4) received subcutaneous injections of DAB_{486}-IL-2 (0.5 mg/kg), over the upper back, the animals in group II (bold line in Fig. 4) received subcutaneous injections of DA(197)B_{486}-IL-2 (0.5 mg/kg) over the upper back, and the animals in group III (dotted line in Fig. 4) received subcutaneous injections of tris buffered saline (TBS) over the upper back. On Day 0 the animals in all groups were immunized with Complete Freund's Adjuvant administered intradermally at several sites on the lower back.

The progression of disease in the animals is shown in Fig. 4. By day 11 the animals exhibited signs of arthritis. The severity of the disease was measured using a scoring system that assigned a score of zero to

four for each of four paws. The maximum index per animal is sixteen. Each animal was scored by two individuals. The individual scores were averaged to give a final arthritis index for an animal. A mean score per group is used to describe the condition of a group. The TBS control animals were euthanized on day 18 because their arthritis had become so severe that it was very difficult for them to move to obtain food and water. In contrast, the DAB_{486}-IL-2 and the $DA(197)B_{486}$-IL-2 treated animals had only moderate disease. After the initial peak in mean arthritic score (day 20), the DAB486-IL-2 and $DA(197)B_{486}$-IL-2 treated groups displayed a mean score of between 2 and 5. On day 62 the DAB_{486}-IL-2 and CRM197-IL2 treated groups continued to show similar clinical signs and similar scores.

$DA(197)B_{486}$-IL-2 Induces Immunologic Unresponsiveness

The ability of $DA(197)B_{486}$-IL-2 to induce a state of immunologic unresponsiveness in vivo to a hapten introduced during $DA(197)B_{486}$-IL-2 treatment was measured in a two phase delayed type hypersensitivity (DTH) response to TNBS. Concomitant with initial immunization with TNBS, mice were treated with either $DA(197)B_{486}$-IL-2 or DAB_{486}-IL-2, as described below.

Treatment began on the day of priming with TNBS and continued through the sixth day. As shown in Fig. 5, untreated control mice (cross hatched bar) mounted a brisk DTH response to TNBS (39 ± 6 U). Mice treated with DAB_{486}-IL-2 (50 ug/d) (open bar) had a marked reduction (23% of positive control) in responsiveness to TNBS (9 ± 2 U). Surprisingly, upon rechallenge of mice treated with $DA(197)B_{486}$-IL-2 (50 ug/d) (checked bar), responsiveness to TNBS was suppressed to 54% (21 ± 5 U), compared to untreated control.

DAB_{486}-IL-2 (Seragen, Inc.) has been shown to be immunosuppressive in murine model of DTH using a single hapten (TNBS), Kelley et al. (1988) Proc. Natl. Acad. Sci. USA 85:3980-3984, hereby incorporated by reference. DA(197)B_{486}-IL-2 and DAB_{486}-IL-2 were purified by the same method from cellular extracts of Escherichia coli. Both hybrid protein preparations were essentially free of contamination by endotoxins and were diluted in tris buffered saline (TBS), pH 7.9. In designing these experiments, CRM197-Il-2 was not expected to suppress the DTH response, and therefore was intended to be a negative control molecule for DAB_{486}-IL-2 (Bastos, M.G. et al., J. of Immunol. in press).

Male, 6-8 week old BALB/c ByJ mice (Jackson Laboratory, Bar Harbor, Maine) were primed with 10mM solution of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (ICN Pharmaceuticals, Inc., Cleveland, OH) in sterile PBS by subcutaneous injection, bilaterally into the dorsum. Six days after priming, these mice were challenged with 30μl of 10mM TNBS into the right footpad. Twenty-four hours after challenge, the thickness of both footpads was measured using a micrometer (Starrett, Athol, MA). Results were expressed in DTH units (U) defined as a difference of 0.001mm in thickness of injected and uninjected footpads. Mice received either no treatment, or daily subcutaneous injections of DA(197)B_{486}-IL-2 or DAB_{486}-IL-2, DTH responses were measured in a blinded fashion by individuals lacking knowledge of the treatment protocol in the test host.

CRM197-Induced Immunologic Unresponsiveness is Hapten Specific

To test whether the unresponsiveness to antigen induced by treatment with DA(197)B_{486}-IL-2 was specific for the immunizing antigen or due to induction or generalized immunosuppression, mice that had been

immunized with TNBS during DA(197)B_{486}-IL-2 therapy were immunized with a second, non-crossreactive hapten, DNFB, after cessation of DA(197)B_{486}-IL-2 therapy. If DA(197)B_{486}-IL-2 selectively targets antigen activated clones, then DA(197)B_{486}-IL-2 treatment during the first phase response to TNBS should spare reactivity to DNFB during the second phase, since this hapten was introduced after cessation of DA(197)B_{486}-IL-2 therapy.

The following experiments illustrate the specificity of the immunosuppressive effect of DA(197)B_{486}-IL-2. One to four weeks after the initial DTH response to TNBS was obtained, the same mice were re-exposed to TNBS. Simultaneously, the mice were immunized with a second, non-cross-reactive hapten, 2,4-dinitro-1-fluorobenzene (DNFB) (Sigma Chemical Company, St. Louis, MO), by two consecutive daily abdominal paintings of 0.5% DNFB (25ul) diluted in a 4:1 acetone/olive oil mixture (vol:vol). Four days after the last abdominal painting, mice were re-exposed to 0.2% DNFB (20ul) diluted in acetone/olive oil applied to both ears. DTH response was determined as the difference in ear thickness as measured by micrometer before and 24 hours after ear painting. As shown in Fig. 6 responses to rechallenge by TNBS were much the same upon re-exposure as they had been initially, with a brisk response by the untreated control ($56^{+}_{-}7$U) (cross hatched bar), a highly suppressed response in DAB_{486}-IL-2-treated animals ($9^{+}_{-}2$U) (open bar), and a response midway between these two groups in animals treated with DA(197)B_{486}-IL-2 ($29^{+}_{-}8$U) (checked bar). As shown in Fig. 7, all three groups of animals mounted normal responses to DNFB, suggesting that the immunosuppression seen animals treated with DA(197)B_{486}-IL-2, like that seen in DAB_{486}-IL-2-treated animals, was antigen-specific. In Fig. 7 the cross hatched bar represents treatment with TBS (control), the

checked bar represents treatment with DA(197)B_{486}-IL-2, and the filled bar represents treatment with DAB_{486}-IL-2.

DA(197)B_{486}-IL-2 Stimulates Proliferation of IL-2 Receptor Bearing Cells in Vitro

DAB_{486}-IL-2 has been shown to significantly inhibit protein synthesis and proliferation in IL-2 receptor (IL-2R) bearing cells by virtue of its ability to catalyze the ADP-ribosylation of elongation factor 2, see Bacha et al. (1988) J. Exp. Med. 167:612, hereby incorporated by reference. The gly-glu alteration in primary sequence at position 53 in DA(197)B_{486}-IL-2 inactivates the ADP-ribosylating activity of the toxophore without substantially altering its IL-2 receptor binding characteristics. We therefore were interested in knowing whether the modified fusion toxin retained any IL-2 like character. The biologic activity of DA(197)B_{486}-IL-2 was addressed in experiments designed to measure its effects on the uptake of [3H]-thymidine (an index of cellular DNA synthetic and proliferative capacity) in peripheral blood mononuclear cells (PBMC).

Although not equivalent on a mole per mole basis, see Fig. 8, DA(197)B_{486}-IL-2 was capable of stimulating significant incorporation of [^{3}H]-thymidine in mitogen-activated PBMC. In Fig. 8 increasing doses of IL-2 (filled circles) and DA(197)B_{486}-IL-2 (open circles) were added to human PBMC that had been activated for 72 h with phytohemagglutinin (PHA) (Difco, Detroit, MI). Cultures were pulsed after 20 h with ^{3}H-thymidine and levels of incorporation of label determined. The ED_{50} for rIL-2, the point at which uptake of label was half-maximal, was approximately 9 X 10^{-11}M; about 30-fold more DA(197)B_{486}-IL-2 than IL-2 was required to half-maximally

stimulate [^{3}H]-thymidine incorporation by the same cell population (IL-2 ED_{50} ~ 3 X 10^{-9}M).

PBMC were isolated from the peripheral blood of normal healthy volunteers by Ficoll-Hypaque density gradient centrifugation. For activation, cells were resuspended to 1 X 10^6/ml in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 25mM HEPES, pH 7.4, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS, Gibco) to which 5-10 µg/ml phytohemagglutinin (PHA, Difco, Detroit, MI) was added. After 72h, cells were washed three times with medium and seeded at 10^5/well into 96 well V-bottom microtiter trays (Nunc. Roskilde, Denmark) in medium containing either rIL-2 or DA(197)B_{486}-IL-2. Subsequent to a 20h incubation with these compounds, each culture was pulsed with 2.5 µCi [^{3}H]-thymidine (70-90 Ci/mmol, New England Nuclear, Boston, MA) for 2 hours then collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters were washed, dried and counted on a Beckman LS5000 TD liquid scintillation counter.

Binding Characteristics of DA(197)B_{486}-IL-2

The IL-2 receptor binding characteristics of DAB_{486}-IL-2 and DA(197)B_{486}-IL-2 were compared in a series of experiments using high affinity IL-2R bearing HUT 102/6TG cells and intermediate affinity IL-2R bearing YT2C2 cells. As shown in Table 2, the high affinity IL-2R binding constant (K_d) of the DA(197)B_{486}-IL-2 molecule was comparable to that determined for DAB_{486}-IL-2; approximately 3 X 10^{-9}M in the former case and 1.5 X 10^{-9}M in the latter. Table 2 also shows that DA(197)B_{486}-IL-2 displayed a K_d for the intermediate affinity IL-2R on YT2C2 cells which again was comparable

to that of DAB_{486}-IL-2, about 7 X 10^{-7}M for CRM 197-IL-2 vs. 3.5 X 10^{-7}M for DAB_{486}-IL-2.

DAB_{486}-IL-2, $DA(197)B_{486}$-IL-2 and rIL-2 were iodinated using enzymobeads (BioRad, Richmond, CA) according to the instructions of the manufacturer. Six hundred to one thousand μCi of [^{125}I] Na (Amersham, Arlington Heights, IL) was added to approximately 25 μg of DAB_{486}-IL-2, rIL-2 or 22 μg of $DA(197)B_{486}$-IL-2, 50 μl 0.2M sodium phosphate pH 7.2, 50μl enzymobeads, and 10 μl 5% β-D-glucose. The reaction was typically allowed to proceed for 5-10 min at room temperature, whereupon 15 μl 0.25% NaN_3, and 15 μl 200mM B-mercaptoethanol were added and the reaction centrifuged at 14,000g for 30 sec. The reaction was removed, diluted to 750 μl with RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 25mM HEPES, pH 7.4, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS, Gibco). The diluted reaction mixture was then applied to a 10ml Bio-Gel P-6 DG column (BioRad). Specific activities of [^{125}I] DAB_{486}-IL-2, [^{125}I] rIL-2, and [^{125}I] $DA(197)B_{486}$-IL-2 were approximately 23-35 μCi/μg.

Measurements of equilibrium binding constants were made for [^{125}I] DAB_{486}-IL-2 and [^{125}I] $DA(197)B_{486}$-IL-2 essentially as described by Wang and Smith with modifications, see Wang et al. (1987) J. Exp. Med. 166:1055, hereby incorporated by reference. High affinity Il-2 receptor-expressing HUT102/6TG cells were harvested and washed thoroughly in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 25mM HEPES, pH 7.4, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS (Gibco) prior to assay. Five hundred to seven hundred thousand cells (0.1 ml) were added to increasing concentrations of either radiolabeled DAB_{486}-IL-2 or $DA(197)B_{486}$-IL-2. The cell suspension was overlayed on a mixture of 80% 550 Fluid (Accumetric, Inc.

Elizabethtown, KY); 20% light mineral oil (Sigma, St. Louis, MO) and incubated 30 min at 37°C under 5% CO_2. The incubation mixture was microcentrifuged and the cell pellet (bound ligand) severed and counted separately from the supernatant (free ligand) in a Nuclear Chicago gamma counter. Nonspecific binding was determined in the presence of a 300 to 500 fold molar excess of unlabeled DAB_{486}-IL-2 or DA(197)B_{486}-IL-2 and represented approximately 10-20% of total cpm bound. In some experiments with [^{125}I] DA(197)B_{486}-IL-2, a 100-fold molar excess of unlabeled rIL-2 was used. Specific cpm bound were calculated and plotted according to the method of Scatchard, see Scatchard et al. (1949) Ann. New York Acad. Sci. 51:660, hereby incorporated by reference.

Because of the higher concentrations of ligand required, the intermediate affinity binding constant determinations were performed using competitive displacement assays. In these studies, HUT 102/6TG or YT2C2 cells were harvested and washed three times with RPMI 1640 medium containing 25 mM HEPES, pH 7.4, 2mM L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin and 10% FCS. Cells were added at 5 X 10^6 - 14 X 10^6/ml to [^{125}I]rIL-2 in the presence or absence of increasing concentrations of unlabeled rIL-2, DAB_{486}-IL-2 or DA(197)B_{486}-IL-2. The cell suspension was then incubated for 30-120 min at 37° under 5% CO_2. In some experiments cells were pretreated at 37°C for 60 min in phosphate buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA) (Sigma, St. Louis, MO), 15 mM NaN_3, and 50 mM 2-deoxy-D-glucose, pH 7.2, to inhibit internalization of radiolabeled ligand, see Ciechanover et al. (1983) J. Biol. Chem. 258:9681, hereby incorporated by reference. The reaction was then overlayed on a mixture of 80% 550 fluid (Accumetric Inc., Elizabethtown, KN); 20% paraffin oil (p = 1.03 g/ml) and microcentrifuged, after which the

cell pellet was excised. The aqueous phase and pellet of each sample, representing free and bound ligand, respectively, was then counted in a Nuclear Chicago gamma counter. The apparent dissociation constants given above are calculated from the concentrations of unlabeled ligand required to displace 50% of the labeled ligand, viz., [^{125}I]-rIL-2, see Ruffolo (1982) J. Auton. Pharmac. 2:277, hereby incorporated by reference and Waters et al. (1990) Eur. J. Immunol. 20:785, hereby incorporated by reference.

DA(197)B_{486}-IL-2 Does Not Possess Nuclease Activity

In order to explain the paradoxical results obtained in vivo with DA(197)B_{486}-IL-2, we explored the possibility that the molecule may have enzymatic or inhibitory properties which would not be apparent over the course of a 24 hour in vitro assay. In this regard, Chang et al. (1989) Science 246:1165 recently reported the existence of an apparent nuclease activity associated with diphtheria toxin sequences other than those mediating its ADP ribosyltransferase function. We therefore performed the following studies in vitro to determine whether DA(197)B_{486}-IL-2 had biologically significant nuclease activity.

In one set of experiments, normal, human PHA-activated PBMC or HUT 102/6TG cells, both of which express high affinity IL-2R, were treated with DA(197)B_{486}-IL-2 for extended periods of time. DNA synthesis ([^{3}H]-thymidine incorporation) and protein synthesis ([^{3}H]-leucine incorporation were measured at 24 hour intervals for a period of 72-96h following the addition of DA(197)B_{486}-IL-2 to the system. In both the normal and tumor cells studied, stimulation indices (ratio of cpm incorporated in treated vs. untreated cultures) were typically $\geq$ 1.00 (within experimental error) for DNA synthesis and protein synthesis. The lack

of a consistent downward trend in either stimulation index indicated no significant inhibition of either measured function.

In a second set of experiments, HUT 102/6TG cells were treated with 10^{-7}M or 10^{-10}M CRM-197-IL-2 for 24h and DNA prepared for agarose gel electrophoresis and ethidium bromide staining. No obvious degradation of DNA was found in these preparations, indicating no evidence for a nuclease-like activity associated with the DA(197)B_{486}-IL-2 molecule.

Human Dosage and Administration

Dosages of the hybrid proteins of the invention will vary, depending on factors such as the condition of the patient. Generally, the hybrid protein of the invention will be administered by intravenous infusion over a period of one to six hours, in a series, e.g., two to fifteen, more preferably five to ten infusions, given, e.g., once or twice daily or every two or three days, or in regular courses interrupted by periods of cessation of treatment. In the case of transplants treatment will begin on the day of the transplant; each dose preferably will be in the range of about 0.2-1.0 mg/kg. See Strom U.S.S.N. 772,893, filed September 5, 1985, hereby incorporated by reference, for guidance in timing treatments with proliferative bursts. In some instances, treatment initiation can be delayed one or more days following the allograft, since therapy not only can prevent rejection, but can reverse it as well.

Other embodiments are within the following claims.

TABLE I AUTOIMMUNE DISEASE AND THEIR CHARACTER AUTOANTIGENS

DISEASE	TARGET OF ANTIBODY
Organ-Specific Diseases	
Juvenile insulin-dependent diabetes	Pancreatic islet cells insulin
Insulin-resistant diabetes with acanthosis nigricans	Insulin-receptor
Insulin-resistant diabetes with ataxia telangiectasia	Insulin receptor
Myasthenia gravis	Acetylcholine receptors
Graves' disease	Thyroid-stimulating hormone receptor
Thyroiditis	Thyroid
Allergic rhinitis, asthma, and autoimmune abnormalities	$Beta_2$-adrenergic receptors
Pernicious anemia	Gastric parietal cells, vitamin B_{12} binding site of intrinsic factor
Addison's disease	Adrenal cells
Idiopathic hypoparathyroidism	Parathyroid cells
Spontaneous infertility	Sperm

Premature ovarian failure	Interstitial cells, corpus luteum cells
Pemphigus	Intercellular substance of skin and mucosa
Bullous pemphigoid mucosa	Basement membrane zone of skin and
Primary biliary cirrhosis	Mitochondria
Autoimmune hemolytic anemia	Erythrocytes
Idiopathic neutropenia	Platelets
Idiopathic neutropenia	Neutrophils
Vitiligo	Melanocytes
Osteosclerosis and Meniere's disease	Type II collagen
Chronic active hepatitis	Nuclei of hepatocytes

Systemic (Non-Organ-Specific) Diseases	
Rheumatoid arthritis	Gamma globulin, Epstein-Barr virus-related antigens, II and III collagen types
Systemic lupus erythematosus	Nuclei, double-stranded DNA, SM, single-stranded DNA, ribonucleo-protein, lymphocytes erythrocytes, neurons, gamma globulin
Goodpasture's syndrome	Basement membranes
Sjogren's syndrome SS-	Gamma globulin, SS-A (Ro), B (La)
Scleroderma SS-	Nuclei, SCL-70, SS-A (Ro), B (La), centromere
Polymyositis	Nuclei, Jo-1 PL-7, histadyl-tRNA synthetase, threonyl-tRNA synthetase, PM-1, Mi-2
Rheumatic fever	Myocardium, heart valves, choroid plexus

Table 2

Receptor binding characteristics of $DA_{(197)}B_{486}IL\text{-}2$ and $DAB_{486}IL\text{-}2$

	K_d(M)	
Compound	High Affinity	Intermediate Affinity
$DA_{(197)}B_{486}IL\text{-}2$	3.0×10^{-9}	7×10^{-7}
$DAB_{486}IL\text{-}2$	1.6×10^{-9}	3.5×10^{-7}

CLAIMS

1. A hybrid protein comprising protein fragments joined together by covalent bonds, said protein fragments comprising,

(a) a fragment comprising a portion of IL-2, said portion including at least a portion of the binding domain of IL-2, said portion of said binding domain being effective to cause said hybrid protein to bind selectively to cells bearing the IL-2 receptor, and

(b) an enzymatically inactive fragment of diphtheria toxin which does not include a functional diphtheria toxin generalized eukaryotic binding site,

said hybrid protein capable of stimulating the proliferation of PBMC *in vitro* and of suppressing an immune response in a mammal *in vivo*.

2. The hybrid protein of claim 1, wherein said portion of the diphtheria toxin molecule has been enzymatically inactivated by a mutation.

3. The hybrid protein of claim 2, where said mutation is at position 53 of the diphtheria toxin.

4. The hybrid protein of claim 1, wherein said inactive fragment comprises residues 1-485 of diphtheria toxin.

5. The inactive fragment of claim 1, wherein domain 1_2 of diptheria toxin is deleted.

6. The inactive fragment of claim 1, wherein the hydrophobic transmembrane region of diphtheria toxin is deleted.

7. The inactive fragment of claim 1, wherein Fragment A of diphtheria toxin is deleted.

8. The inactive fragment of claim 1, wherein Fragment B of diphtheria toxin is deleted.

9. The inactive fragment of claim 1, wherein the protease sensitive domain of diphtheria toxin is deleted.

10. The inactive fragment of claim 1, wherein at least 10% of the amino acid residues are deleted.

11. The inactive fragment of claim 1, wherein at least 20% of the amino acid residues are deleted.

12. The inactive fragment of claim 1, wherein at least 30% of the amino acid residues have been deleted.

13. A DNA sequence encoding the hybrid protein of claim 1.

14. An expression vector containing the DNA sequence of claim 13.

15. A cell transformed with the vector of claim 14.

16. A method of producing the hybrid protein of claim 1 comprising culturing the cell of claim 15, and isolating said hybrid protein from the cultured cell or supernatant.

17. A method of inhibiting an unwanted immune response in a mammal comprising administering to said

mammal an inhibiting amount of the hybrid protein of claim 1.

18. A method of inhibiting the T-lymphocyte-induced rejection of an allograft in a mammal comprising administering to said mammal, following said allograft, and during said proliferative burst, the hybrid molecule of claim 1.

19. A method of treating a patient having a disease characterized by a proliferative burst of lymphocytes comprising administering to said patient, during said proliferative burst, the hybrid protein of claim 1.

20. The method of claim 19, wherein said disease is an autoimmune disease.

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ATG GCA CCT ACT TCT AGC TCT ACC AAG AAA ACC CAG CTG CAG CTC GAG
Met Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln Leu Glu
1               5                  10                  15

CAC CTG CTG CTG GAT TTG CAG ATG ATC CTG AAC GGT ATC AAC AAT TAC
His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr
            20                  25                  30

AAG AAC CCG AAA CTG ACG CGT ATG CTG ACC TTC AAG TTC TAC ATG CCG
Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro
        35                  40                  45

AAG AAG GCC ACC GAA CTG AAA CAC CTG CAG TGT CTA GAA GAA GAA CTG
Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu
    50                  55                  60

AAA CCG CTG GAG GAA GTT CTG AAC CTG GCT CAG TCT AAA AAC TTC CAC
Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe His
65                  70                  75                  80

CTG CGG CCG CGT GAC CTG ATC TCT AAC ATC AAC GTA ATC GTT CTG GAA
Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val Leu Glu
                85                  90                  95

CTG AAG GGC TCT GAA ACC ACC TTC ATG TGT GAA TAC GCT GAT GAG ACC
Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr
            100                 105                 110

GCA ACC ATC GTA GAA TTC CTG AAC CGT TGG ATC ACC TTC TGT CAG TCT
Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser
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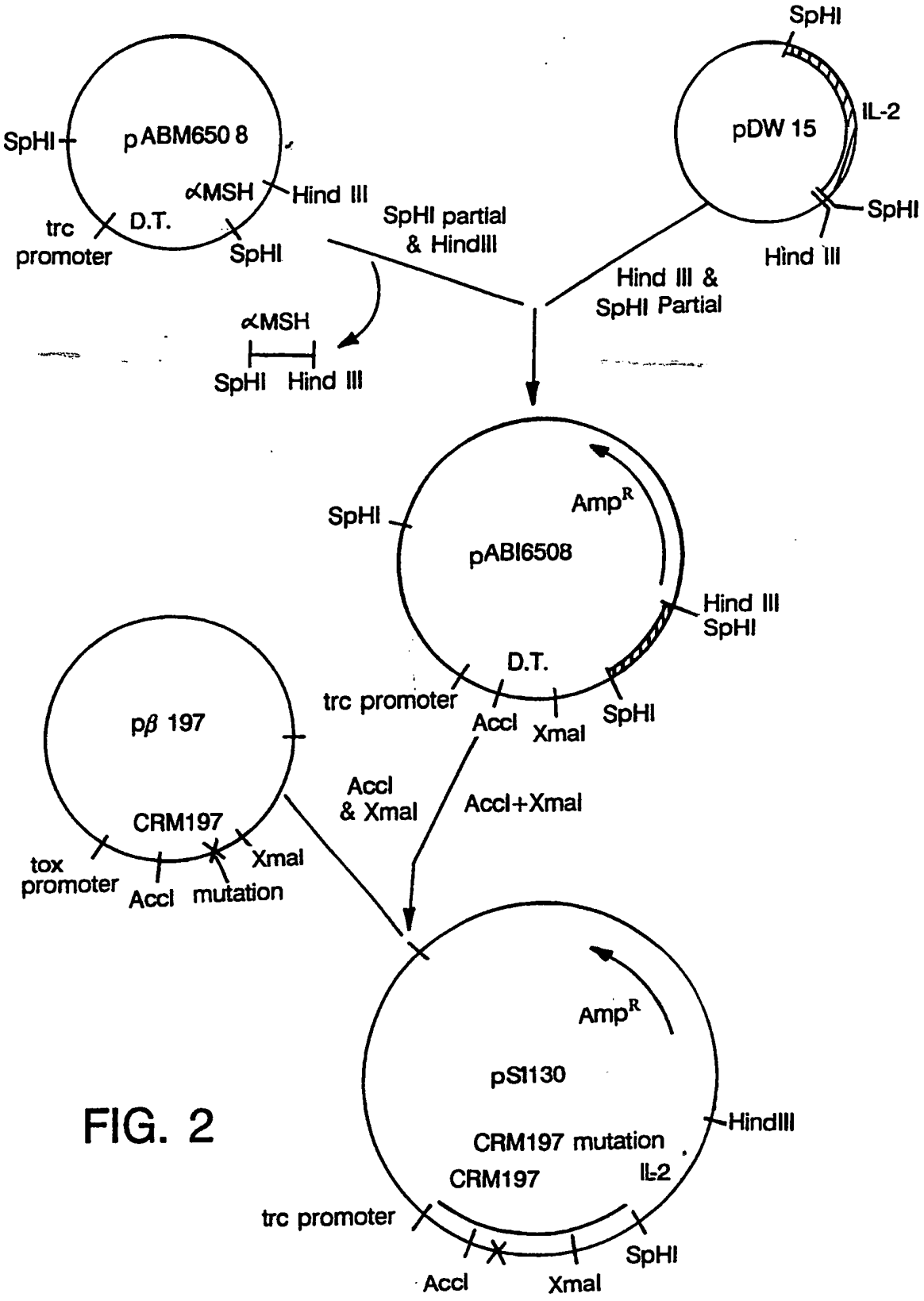
ATC ATC TCT ACC CTG ACC
Ile Ile Ser Thr Leu Thr
    130
```

FIG. 1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SpHI
αMSH
Hind III
trc
promoter
D.T.
SpHI
SpHI partial
& HindIII
αMSH
SpHI Hind III
SpHI
pDW 15
IL-2
SpHI
Hind III
Hind III &
SpHI Partial
SpHI
pABI6508
Amp^R
Hind III
SpHI
D.T.
trc promoter
AccI
XmaI
SpHI
pβ 197
CRM197
tox
promoter
AccI
mutation
XmaI
AccI
& XmaI
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FIG. 2

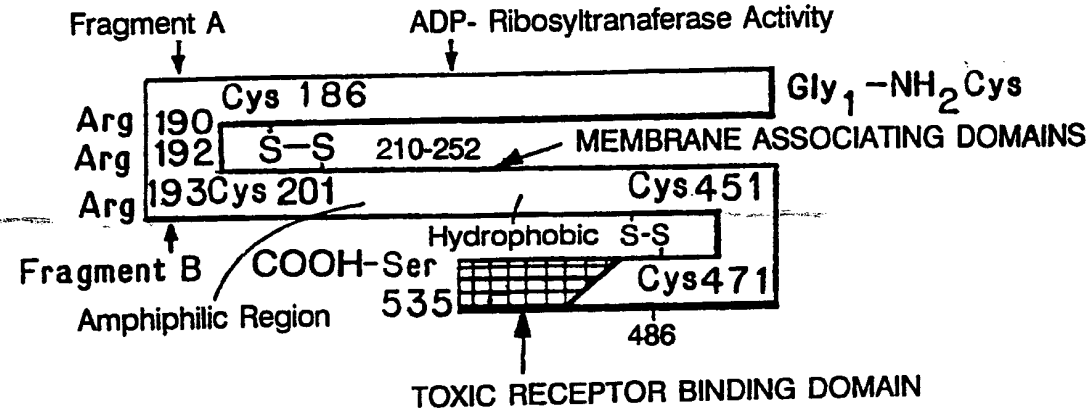

Fragment A
ADP- Ribosyltranaferase Activity
Cys 186
Gly1 –NH2 Cys
Arg 190
Arg 192
S–S 210-252
MEMBRANE ASSOCIATING DOMAINS
Arg 193 Cys 201
Cys 451
Hydrophobic S-S
Fragment B
COOH-Ser 535
Cys 471
Amphiphilic Region
486
TOXIC RECEPTOR BINDING DOMAIN

FIG. 3

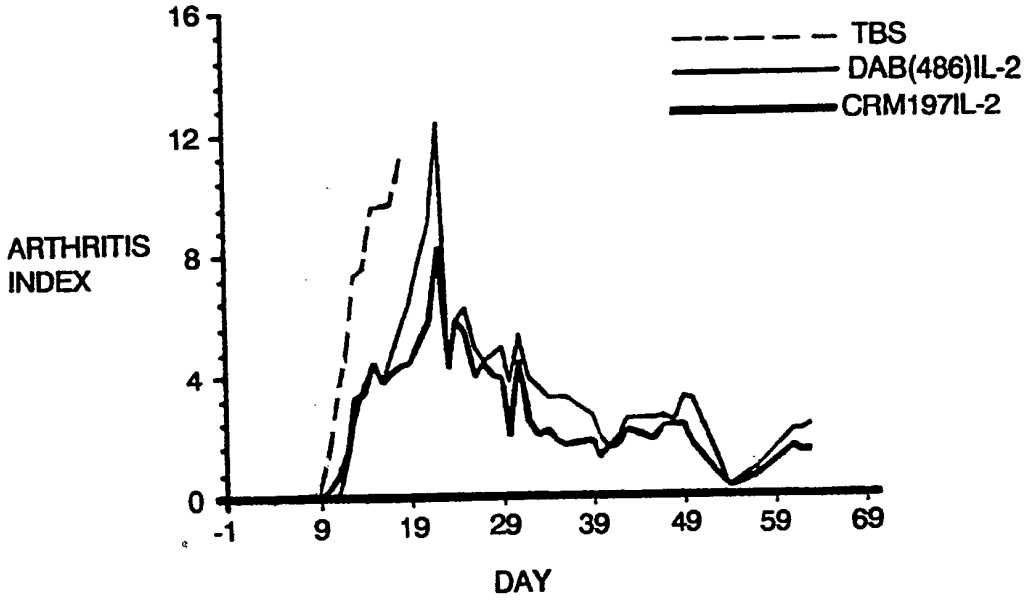

TBS
DAB(486)IL-2
CRM197IL-2
ARTHRITIS INDEX
16
12
8
4
0
-1
9
19
29
39
49
59
69
DAY

FIG. 4

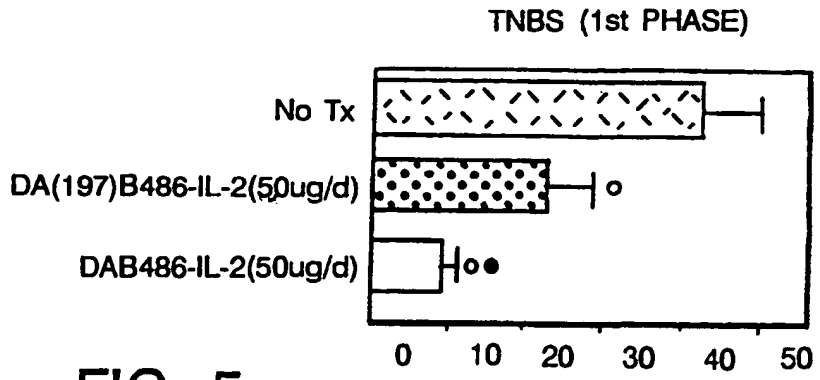


FIG. 5

o p<0.001 vs NoTx
● p<0.05 vs DA(197)B486-IL-2

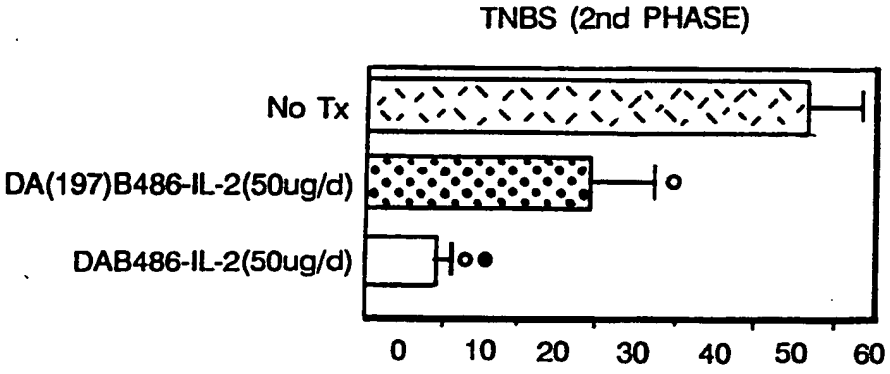


FIG. 6

o p<0.001 vs NoTx
● p<0.05 vs DA(197)B486-IL-2

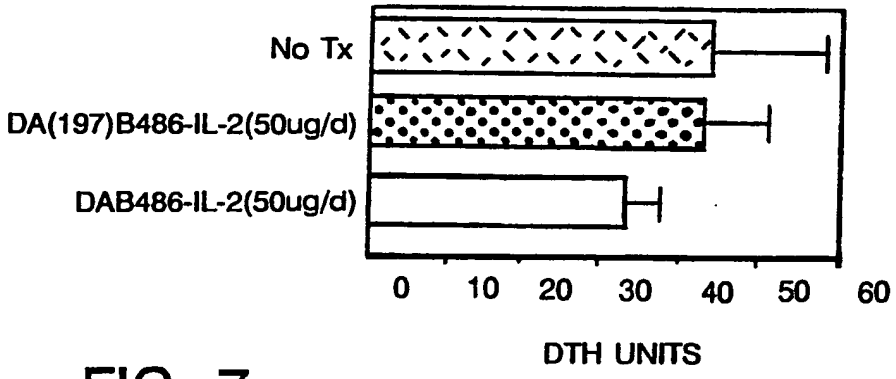
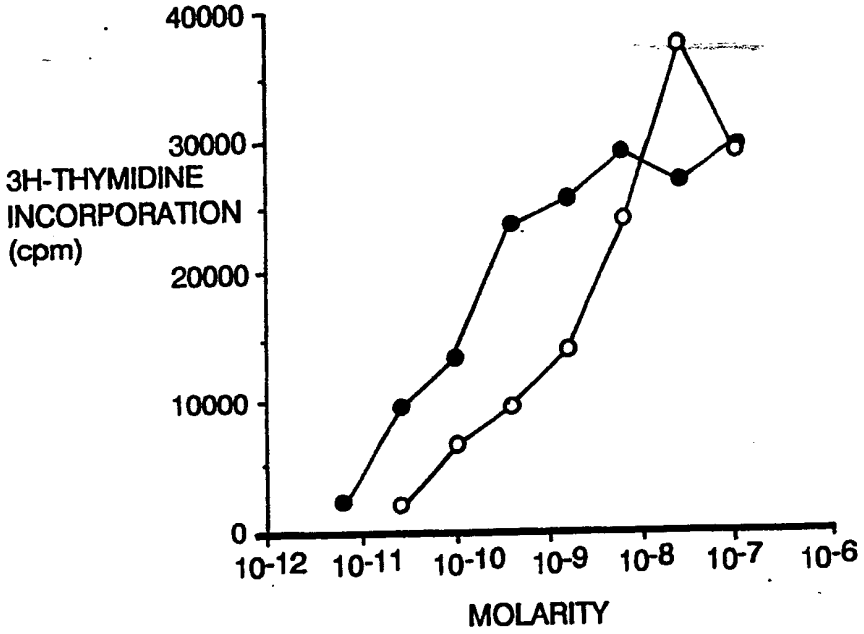


FIG. 7

o p<0.001 vs NoTx
● p<0.05 vs DA(197)B486-IL-2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INCORPORATION
(cpm)
20000
10000
0
10-12
10-11
10-10
10-9
10-8
10-7
10-6
MOLARITY

FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07342

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 15/00; C12P 21/00; C12N 15/24, 15/70; 5/10; C07H 15/12; A61K 37/02

U.S.Cl: 530/351; 435/69.5, 69.52, 69.7, 172.3, 320.1; 536/27; 424/85.2, 85.91

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl:	530/351; 435/69.5, 69.52, 69.7, 172.3, 240.1, 243, 252.3, 320.1; 536/27; 424/85.2, 85.91; 930/141, 200; 935/11, 22, 47, 107

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Eur. J. Immunol, vol. 20, issued April 1990 Waters et al, "Interleukin 2 Receptor-targeted cytoxicity. Receptor binding requirements for entry of a Diphtheria Toxin-related Interleukin 2 fusion protein into cells", pages 785-91. see entire document.	1-2,4, 10-17,19 1-19
X Y	Protein Engineering, vol. 1, No. 6, issued 1987, Williams et al, "Diphtheria toxin receptor binding Domain substitution with Interleukin-2: Genetic construction and properties of a Diphtheria Toxin-Related Interleukin-2 fusion protein" pages 493-98, see entire document.	1-2,4, 10-17,19 1-19

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 December 1991	13 DEC 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	for Garnette D. Draper, Prim. Exm.

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	Transplantation, vol. 47, no 2, issued February 1989, Kirtman et al, "Prolongation of cardiac allograft survival in Murine Recipients Treated with a Diphtheria Toxin-Related Interleukin-2 fusion protein 32", pages 327-330, see entire document.	1-2,4, 10-16,18 1-19
X Y	Proc. Natl. Acad. Sci., 85, issued June 1988, Kelly et al, "Interleukin 1-Diphtheria Toxin Fusion Protein can abolish cell-mediated Immunity in vivo", pages 3980-84, see entire document.	1-2,4 10-17,19 1-19
Y	Proc. Natl. Acad. Sci., Vol. 85, issued November 1988, Perentesis et al, "Expression of Diphtheria Toxin Fragment A and Hormone-Toxin Fusion Protein in Toxin-Resistant yeast mutants", pages 8386-90, see entire document.	1-19
Y	US, A, 4,675,382, (Murphy), 23 June 1987, see claims.	1-19
A	US, A, 4,935,233 (Bell et al), 19 June 1990.	1-19